REMARKS

Claims 1 and 3 are pending in the instant application. Claims 1 and 3 have been rejected. Claim 1 has been amended. No new matter has been added by this amendment. Reconsideration is respectfully requested in light of the following remarks.

I. Rejection of Claims Under 35 U.S.C. §112

The rejection of claims 1 and 3 under 35 U.S.C. §112, first paragraph, because the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with the claims has been maintained. Specifically, the Examiner suggests that while being enabled for a compound comprising a baculovirus expressed recombinant Fel dI wherein the baculovirus expressed recombinant Fel dI comprises a sFv humanized anti-CD64 monoclonal antibody H22 fused to Fel dI chain 1 and Fel dI chain 2 wherein chain 1 and chain 2 are linked in series by a glycine/serine linker encoded by SEQ ID NO:5 as shown in Figure 1 for diagnosis of cat allergy, the specification does not reasonably enable any compound as set for in claims 1 and 3 for diagnosis and treatment of cat allergy. The Examiner suggests that there is insufficient guidance provided by the specification on the hybridization conditions using primers SEQ ID NO:1-4 for amplifying the nucleic acid sequences that encode the Fel dI chain 1 and chain 2 and that, as evidenced by Wallace *et al.* and Sambrook *et al.*, such specificity in hybridization is empirical by nature and the effect of mismatches within an oligonucleotide probe is unpredictable. Accordingly, the Examiner suggests that the undue experimentation would be required to practice the

claimed invention as there is insufficient guidance as to the structure of nucleic acid sequence encoding Fel dI chain 1 and Fel dI chain 2 expressed in series and linked together by a glycine serine linker.

Applicants respectfully traverse this rejection.

Applicants disagree with the Examiner and maintain that with limited experimentation, one of skill in the art could readily isolate a nucleic acid sequence encoding Fel dI chain 1 and chain 2. As the claims are directed to specifically identified template sequences and specifically identified oligonucleotide probe sequences which are 100% complementary to said template sequence, Applicants believe that the rejection under 35 U.S.C. §112, first paragraph, is improper. The courts have maintained that when claims are directed to any purified and isolated DNA sequence encoding a specifically named protein where the protein has a specifically identified sequence, a rejection of the claims as broader than the enabling disclosure is generally not appropriate because one skilled in the art could readily determine any one of the claimed embodiments. See 27 F.2d at 1213-14, 18 USPQ2d at 1027.

Further, MPEP 2164.03 states that the amount of guidance or direction needed to enable the invention is inversely related to the amount of knowledge in the state of the art as well as the predictability in the art. In re Fisher, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). The more that is known in the prior art about the nature of the invention, how to make, and how to use the invention, and the more predictable the art is, the less information needs to be explicitly stated in the specification. Moreover, if one skilled in the art can readily anticipate the

effect of a change within the subject matter to which the claimed invention pertains, then there is predictability in the art.

The Examiner cites Wallace et al. and Sambrook et al. in establishing that the determination of specificity of hybridization probes is empirical by nature and the effect of mismatches within an oligonucleotide probe is unpredictable. Rogers et al. ((1993) *Mol. Immunol.* 30:559-568, enclosed herewith) teach the construction of Fel dI chain 1 and chain 2 expression vectors pET11chain 1 an pET11 chain 2 (see page 560, column 1, ¶2) using nucleotide sequences disclosed in Figure 3 of Morgenstern et al. ((1991) *Proc. Natl. Acad. Sci. USA* 88:9690-9694, enclosed herewith). As evidenced by the combined teachings of Morgenstern et al. and Rogers et al., Applicants respectfully point out that the oligonucleotides probes disclosed in the instant application are 100% homologous with the disclosed plasmid templates: nucleotides 12-32 of chain 1 forward primer (page 4 of the instant application) are 100% complementary with nucleotides 74-94 of Fel dI chain 1 (see, Morgenstern et al., Figure 3A); nucleotides 23-36 of chain 1 reverse primer are 100% complementary with nucleotides 270-283 of Fel dI chain 1 (see, Morgenstern et al., Figure 3A); nucleotides 11-28 of chain 2 forward primer are 100% complementary with nucleotides 59-76 of Fel dI chain 2 (see, Morgenstern et al., Figure 3B); and nucleotides 22-33 of chain 2 reverse primer are 100% complementary with nucleotides 320-330 of Fel dI chain 2 (see, Morgenstern et al., Figure 3B). Therefore, the unpredictability imparted by mismatches is not a factor in the PCR amplification of Fel dI chain 1 and chain 2 when using the primers and templates set forth in claim 1 of the present invention.

Furthermore, it was well-known within the art at the time of filing that a non-degenerate PCR reaction could be carried under annealing temperatures ranging between ~55-70°C (see, for example Peters and Sikorski (June 1998) *Science* 280:1956). With an undue amount of experimentation (~4 PCR reactions, each carried out at annealing temperatures at 5°C intervals), one of skill could readily anticipate amplifying a Fel dI chain 1 or chain 2 fragment given a specifically identified template sequence and specifically identified oligonucleotide primers as, even under suboptimal annealing temperatures, such a highly specific primer/template combination will yield a product albeit possibly with reduced yields under suboptimal conditions.

As the scope of the claims is limited to a specific set of primers used in combination with a specific template, of which the sequence was readily known at the time of filing, Applicants believe that the teachings of the instant specification are commensurate with the scope of the claims and that one of skill in the art could practice the claimed invention without undue experimentation. Withdrawal of this rejection is therefore respectfully requested.

The rejection of claims 1 and 3 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way to reasonably convey to one skilled in the art that the inventor, at the time the application was filed, had possession of the claimed invention has also been maintained. Specifically, the Examiner suggests that the specification does not teach the specific PCR condition using primers 1-4 to amplify any nucleic acid sequences that encoded Fel dI chain 1 and chain 2 and that claim 1 recites more than one

nucleic acid sequences encoding chain 1 and chain 2 which are amplifiable by the specific set of primers. Thus, the Examiner suggests that given the indefinite number of undisclosed nucleic acid sequences and PCR conditions for making the claimed compound, the compounds of claim 1 and, based on dependency, claim 3 are not adequately described. Applicants respectfully traverse this rejection.

As set forth *supra,* one of skill in the art, given the specific primer/template combination disclosed in the instant invention, could readily produce and identify the resulting amplicon as Fel dI chain 1 and chain 2 sequences as these sequences were known in the art at the time of filing and non-degenerate PCR was well-established. The Examiner suggests that claim 1 recites more than one nucleic acid sequences encoding chain 1 and chain 2 which are amplifiable by the specific set of primers. Thus, in an earnest effort to facilitate the prosecution of this application, Applicants have further amended claim 1 to indicate that chain 1 is amplified by PCR using Fel dI chain 1 complementary primers of SEQ ID NO:1 and SEQ ID NO:2, wherein the template for PCR <u>is</u> pET11dΔHR chain-1 *Feld*I; and chain 2 is amplified by PCR using Fel dI chain 2 complementary primers of SEQ ID NO:3 and SEQ ID NO:4, wherein the template for PCR <u>is</u> pET11dΔHR chain-2 *Feld*I. Accordingly, a single set of identified primers are used in combination with a known template to amplify a single product of known sequence in a non-degenerate PCR reaction. In light of this amendment and these remarks, withdrawal of this rejection is respectfully requested.

II. Conclusion

The Applicants believe that the foregoing comprises a full and complete response to the Office Action of record. Accordingly, favorable reconsideration and subsequent allowance of the pending claims is earnestly solicited.

Respectfully submitted,

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1) Combined Amendment & Petition for Extension of Time Under 37 CFR 1.136(a)(Small Entity) Transmittal (2 copies);

2) Reply Under 37 C.F.R. 1.116;

3) Reference: Rogers et al. (1993) *Mol. Immunol.* 30:559-568;

4) Reference: Morgenstern et al. (1991) *Proc. Natl. Acad. Sci USA* 88:9690-9694;

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6) Authorization to charge deposit account in the amount of $55.00.

Jane Massey Licata

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RECOMBINANT *Fel d* I: EXPRESSION, PURIFICATION, IgE BINDING AND REACTION WITH CAT-ALLERGIC HUMAN T CELLS

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Abstract—This study describes the properties of the two recombinantly expressed polypeptide chains of *Fel d* I, the major allergen produced by the domestic cat (*Felis domesticus*). An inframe linker encoding polyhistidine has been added to the 5′ ends of the *Fel d* I chains 1 and 2 cDNAs to facilitate purification using Ni^{2+} ion affinity chromatography. This method provides high yields in a single step of rchain 1 and rchain 2 of *Fel d* I with a >90% level of purity. Polymerase chain reaction (PCR) methods were used to introduce a thrombin cleavage site (LVPR↓GS) at the *N*-terminus of both chains. Thrombin cleavage of rchain 1 and rchain 2 followed by HPLC purification of the cleavage products allowed the isolation of each recombinant chain with only two additional residuals (GS) at the *N*-terminus of the native sequence. Amino acid sequencing analysis of the *N*-terminus and mass spectrometry of these polypeptides demonstrated that they are highly pure and full-length. Direct ELISA assays showed that IgE from cat-allergic patients binds to both rchain 1 and rchain 2 of *Fel d* I, demonstrating that both these chains contribute to the allergenicity of this heterodimeric protein. An examination of the reactivity of T cells derived from cat-allergic patients revealed that both polypeptide chains contribute to the T cell response to this allergen. Consequently, it is concluded that the immunological response to *Fel d* I is composed of a reaction at both the B and T cell level to each of the two chains that constitute the native allergen.

INTRODUCTION

The major allergen derived from the domestic cat (*Felis domesticus*), *Fel d* I (formerly cat allergen 1), is not only clinically significant in terms of allergy to cats (Anderson and Baer, 1981; Ohman *et al.*, 1974; Leitermann and Ohman, 1984; Kaufman and Ranck, 1988) but has also been established as being the agent responsible for cat-induced asthma (Ohman *et al.*, 1984; van Metre *et al.*, 1986). The frequency of cat allergy in the United States is approximately 10% of the general population, making this allergen source clinically significant (Freidhoff *et al.*, 1984). The rapid onset of asthma or rhinitis in allergic patients after exposure has been attributed to the finding that the *Fel d* I protein is found associated with small particles that readily become airborne (Luczynska *et al.*, 1990).

Murine mAbs directed against *Fel d* I have been successfully used to affinity purify the allergen and assess exposure (Chapman *et al.*, 1988; Duffort *et al.* 1988). Immunochemical characterization of affinity purified *Fel d* I established that the allergen is a heterodimeric protein composed of two disulfide linked polypeptide chains (Chapman *et al.*, 1988; Duffort *et al.* 1991; Morgenstern *et al.*, 1991) with apparent M_r of 4000 and 14,000 under reducing conditions on SDS–PAGE (Duffort *et al.*, 1991). Immunohistochemical studies detected the production of *Fel d* I protein in cat salivary and lacrimal glands (Brown *et al.*, 1984; van Milligen *et al.*, 1990) as well as skin sebaceous gland cells (Charpin *et al.*, 1991).

Recently, the *N*-terminal amino acid sequence of the two chains of *Fel d* I were reported (Chapman *et al.*, 1988; Duffort *et al.*, 1991). The determination of 90% of the amino acid sequence of these two chains combined with PCR cloning methods defined the complete primary structure of *Fel d* I (Morgenstern *et al.*, 1991). PCR cloning showed that *Fel d* I is composed of two mature polypeptides of 70 and 92 amino acids, designated chain 1 and chain 2, respectively (Morgenstern *et al.*, 1991; Griffith *et al.*, 1992). Protein sequence determination of affinity purified *Fel d* I (Morgenstern *et al.*, 1991) and PCR analysis of multiple chain 2 cDNAs revealed that there are two distinct forms differing in a small region of sequence near the *C*-terminus (Griffith *et al.*, 1992).

This manuscript describes the high level expression in *E. coli* and purification of rchain 1 and rchain 2 of *Fel d* I. The rchain 1 and 2 fusion proteins have been

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Abbreviations: *Fel d* I, the major allergen from the domestic cat (*Felis domesticus*) named according to the IUIS approved nomenclature [*Ann. Allergy* **60**, 499–504 (1988)]; IPTG, isopropyl-D-thiogalactopyranoside; DTT, dithiothreitol; cp, cleaved and purified; PCR, polymerase chain reaction; TMB, 3,3′,5,5′-tetramethylbenzidine; aa, amino acids; HRPO, horseradish peroxidase; rchain 1, recombinant chain 1 of *Fel d* I; rchain 2, recombinant chain 2 of *Fel d* I; LSM, lymphocyte separation medium.

proteolytically cleaved and HPLC purified to produce recombinant chains largely devoid of non-relevant sequence. The cat allergic human IgE binding capacity of these highly purified chains 1 and 2 have been examined. Furthermore, these recombinant proteins have been used as the stimulating Ag in allergic human T cell proliferation assays *in vitro*. This work clearly demonstrates that both chain 1 and chain 2 significantly contribute to the allergic and immunological properties of *Fel d* I.

MATERIALS AND METHODS

Expression vector construction

The cDNAs encoding chain 1 and chain 2 of *Fel d* I were obtained by PCR cloning of cat salivary gland cDNA (Morgenstern *et al.*, 1991). The 5′-primer, THCH1, and the 3′-primer, FI-8 (Griffith *et al.*, 1992), were used in a PCR (Saiki *et al.*, 1988) with chain 1 cDNA (Morgenstern *et al.*, 1991) to produce a DNA fragment encoding the mature chain 1 sequence with a thrombin cleavage site (LVPR↓GS) (Chang, 1985) introduced at the *N*-terminus (Fig. 1A). A similar PCR was performed with chain 2 cDNA (Morgenstern *et al.*, 1991) and the 5′-primer, THCH2, and the 3′-primer, FII-9, to produce a DNA fragment encoding the mature chain 2 (long form) (Griffith *et al.*, 1992) with a thrombin cleavage site at the *N*-terminus (Fig. 1B). The primers FI-8 and FII-9 encode four *C*-terminal amino acids plus 3′ untranslated sequence of chain 1 and chain 2, respectively (Griffith *et al.*, 1992). Both primers have a *Pst* I recognition sequence at their 5′ ends. Primers THCH1 and THCH2 had an *Eco*R I site at the 5′ end of the oligonucleotides to facilitate insertion of the PCR-derived insert into an *Eco*R *Pst* I digested pTrc99A vector (Amann *et al.*, 1988) that had previously had an oligonucleotide, $(CAC)_6$, encoding six histidine residues, introduced immediately downstream of the *Nco* I cloning site. The resulting *Nco* I–*Hind* III coding sequence was isolated, blunted, had *Bgl* II linkers (pCAGATCTG; New England Biolabs, MA) ligated and the inserts placed into the *Bam* HI site of the expression vector pET-11d (Studier *et al.*, 1990). These expression constructs were designated pET11chain 1 and pET11chain 2, respectively.

Expression and purification of Fel d *I rchain* 1 *and rchain* 2

Expression vectors, pET11chain 1 and pET11chain 2, were transformed into the *E. coli* host strain BL21(DE3) and selected on plates containing 150 mg/ml ampicillin (Studier *et al.*,1990). A single transformant colony was grown up in 2 ml volume of 2 × YT medium containing 150 mg/ml ampicillin at 37°C, for approximately 6 hr. Ten milliliters of this culture was spread onto a selection plate and grown overnight at 37°C. The bacterial lawn was recovered in 2 ml media and added to 500 ml of 4 × YT medium (150 mg/ml ampicillin) and grown at 37°C to $A_{600} = 1.0$ and recombinant expression was induced by the addition of IPTG to a final concn of 1 mM. After 2 hr growth, cells were harvested, lysed and the proteins solubilized in 6 M guanidine HCl buffer containing 100 mM 2-ME as previously described for the ragweed recombinant allergens, *Amb a* I.1 and *Amb a* II (Rogers *et al.*, 1991).

The guanidine HCl lysate containing rchain 1 and rchain 2 was subjected to Ni^{2+} metal-ion affinity chromatography under denaturing conditions in 8 M urea (Hochuli *et al.*, 1988). After elution from the Ni^{2+} chelating support, QIAGEN NTA-Agarose (Diagen GmH, Dusseldorf, Germany), the rchain 1 and rchain 2 protein preparations were dialyzed against PBS to remove urea prior to thrombin cleavage or use in biological assays.

Thrombin cleavage and HPLC purification of rchain 1 *and rchain* 2

Ni^{2+} metal-ion affinity purified rchain 1 and rchain 2 were dialyzed against PBS at 4°C. Thrombin (Boehringer Mannheim) was added to give a final thrombin:rchain 1 ratio of 1:120 (w/w). This cleavage mixture was then incubated at 23°C for 5 hr and the sample then reduced by the addition of guanidine HCl and DTT to give a final concn of 5 M guanidine HCl, 100 mM DTT. This mixture was reduced by incubation at 37°C for 30 min. A sample containing 100 mg of rchain 1 was filtered and then chromatographed on a C_4 column using a Rainin HPLC system and a solvent gradient (solvent A, 0.1% TFA/H_2O; solvent B, TFA/acetonitrile). Collected fractions were assessed by SDS–PAGE and rchain 1cp (where cp signifies cleaved and purified) containing fractions were pooled and the structural integrity of the polypeptide was verified by *N*-terminal sequence analysis and mass spectrometry. Recombinant chain 2 was treated in the same manner except that the thrombin digestion was done in 50 mM Tris buffered 1 M urea to enhance the solubility of the rchain 2 polypeptide.

Protein analysis by N*-terminal sequencing and mass spectrometry*

Protein sequence analysis of cleaved and HPLC purified rchain 1cp and rchain 2cp were performed using an Applied Biosystems (Foster City, CA) model 477A gas-phase sequenator with on-line phenylthiohydantoin derivative analysis (model 120). The mol. wt of the rchain 1cp and rchain 2cp were measured with a matrix-assisted laser-desorption time-of-flight spectrometer (LaserTec Benchtop, Vestec Corporation, Houston, TX). The appropriate dilutions of purified proteins were mixed with an equal volume of matrix, sinapinic acid, to a final concn of 5 μM.

Direct binding ELISA for cat-allergic human IgE

Corning plates were coated with 10 μg/ml of each coating antigen; affinity purified *Fel d* I (Chapman *et al.*, 1988), rchain 1cp and rchain 2cp in PBS at 50 μl/well, and incubated overnight at 4°C. In the ELISA assay, the unbound coating antigens were removed and the wells

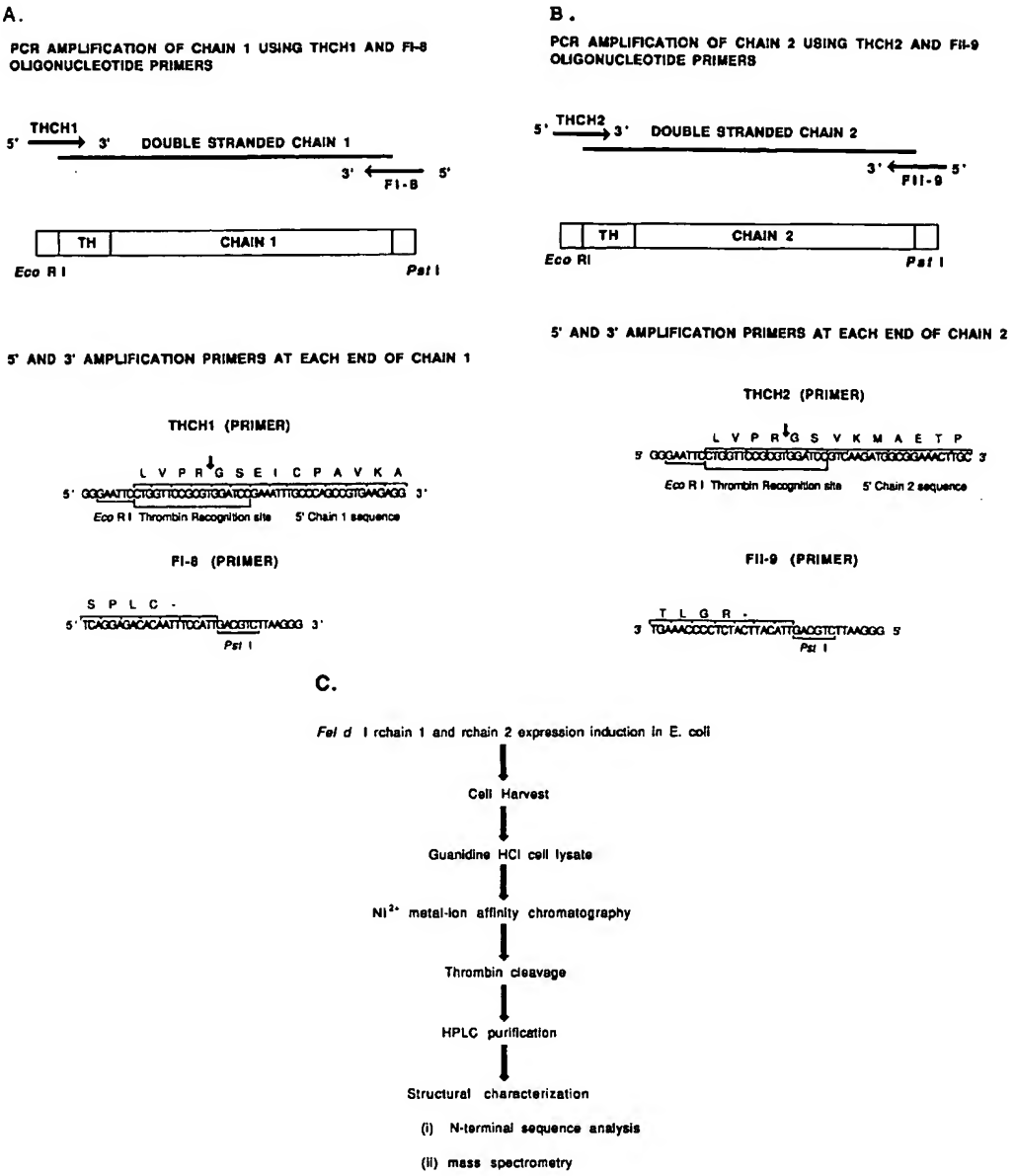

Fig. 1. Expression vector construction and purification scheme of rchain 1 and rchain 2 of *Fel d* I. PCR with specific 5′ and 3′ oligonucleotide primers was used to construct an expression cassette encoding rchain 1 (A) and rchain 2 (B). The single letter code representing encoded amino acids is used. The cDNAs encoding chain 1 and chain 2 used as template for the PCR have been previously described (Morgenstern *et al.*, 1991). The chain 2 cDNA is the long form (Griffith *et al.*, 1992).

In panel (C) the purification scheme for rchain 1 and rchain 2 is schematically outlined.

were blocked with 0.5% gelatin in PBS, 200 μl/well for 2 hr at room temp.

The antibody solution was either an individual plasma or a pool of plasma from patients that were skin test positive for commercial cat extract. Plasma were serially diluted with PBS–Tween 20 (PBS with 0.05% non-ionic detergent Tween-20) (Sigma, St. Louis, MO) and 100 μl/well was added and incubated overnight at 4°C (plasma dilutions were tested in duplicate). This plasma pool had been depleted of most of the IgG antibodies by extraction with Protein G-Agarose (GammaBind G-Agarose, Genex Corp. Gaithersburg, MD). The second antibody (biotinylated goat anti-Human IgE. 1:1000, Kirkgaard & Perry Laboratories, Inc., Gaithersburg, MD), was added at 100 μl/well for 1 hr at room temp. This solution was removed and streptavidin–HRPO, 1:1000, (Southern Biotechnology Associates, Inc., Birmingham, AL) was then added at 100 μl/well for 1 hr at room temp (all wells were washed three times with PBS–Tween between each incubation step). TMB Membrane Peroxidase Substrate System (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was freshly mixed, and added at 100 μl/well. After the addition of 100 μl/well of 1 M phosphoric acid to stop the reaction,

the plates were read on a microplate EL310 Autoreader (Biotek Instruments, Winooski, VT) with a 450 nm filter. The absorbance levels of duplicate wells were averaged.

Isolation of antigen reactive T cell lines

Patients were chosen who were determined to be cat-allergic through both prick skin testing and clinical history. These patients had not undergone immunotherapy and were not taking any medication that would interfere with the immune response. Heparinized peripheral blood samples were taken from these patients and the leukocytes were purified within 24 hr of the sampling. Briefly, the peripheral blood mononuclear leukocytes (PBL) were separated from the plasma by centrifugation at 150 *g* for 15 min. The plasma was removed and used for antibody analysis. The pelleted cells were restored to their original volume in fetal bovine serum (FBS) and the PBL isolated by either centrifugation with Sepracell-MN (Sepracell Corp., Oklahoma City, OK) or Lymphocyte Separation Medium (LSM) (Organon Teknika, Durham, NC). The cells were washed three times with PBS containing 0.1% BSA, and then resuspended in RPMI-1640 supplemented with 10% heat-inactivated human AB serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 5×10^{-5} M 2-ME and 10 mM HEPES. Aliquots of the isolated PBL from each patient were either stimulated with Ag, frozen in liquid nitrogen or transformed with EBV.

Long term T cell lines were established by stimulation of 1×10^6 PBL/ml in complete medium with 10 μg/mL aqueous affinity purified *Fel d* I (Chapman *et al.*, 1988) for 7–10 days at 37°C in a humidified 5% CO_2 incubator. This amount of *Fel d* I protein was determined to be optimal for the activation of T cells from most cat-allergic patients. The non-adherent T cells were isolated after centrifugation with LSM, washed and cultured in complete medium containing rIL-2 (5 U/ml) and rIL-4 (5 U/ml) (recombinant products, Boehringer-Mannheim, Indianapolis, IN). Cells were cultured until "rested" and no longer responsive to growth factors. At this time the cell line was set up in a secondary proliferation assay (see below). The stimulated cell line was saved by freezing and storage in liquid nitrogen.

Antigen presenting cells (APC)

Two sources of APC have been used in these studies. Secondary T cell cultures were established using autologous γ-irradiated (3500 Rad) PBL as APC. In subsequent stimulations either γ-irradiated PBL (3500 Rad) or γ-irradiated autologous EBV transformed cell lines (up to 25,000 Rad) were used as APC. The EBV transformed cell lines were made by incubation of 3×10^6 PBL with 1 ml of B-59/8 Marmoset cell line (ATCC CRL1612) conditioned medium in the presence of 1 μg/ml PMA at 37°C for 60 min. The cells were then diluted to 5×10^5 cells/ml in medium containing 10% FBS and cultured in 96-well culture plates (Costar, Cambridge, MA) until visible colonies were detected. The EBV cell line was then grown and used for APC.

T cell proliferation assay

T cell lines were used for assay after the rest cycle of their growth. 2×10^4 T cells were cultured in 200 μl of complete medium with γ-irradiated PBL (3500 Rads) (5×10^4) or EBV (25,000 Rads) (2×10^4) cells as APC and with various concns of a panel of Ag in round bottom 96-well dishes (Costar, Cambridge, MA) for 3 days at 37°C in a humidified 5% CO_2 incubator. The cultures were pulsed with tritiated thymidine (1 μCi/well ^{3}H-methyl-thymidine, ICN, Irvine, CA) on day 3 and harvested on glass fiber filters on day 4. The amount of incorporated radioactivity was measured by liquid scintillation counting. Results are expressed as mean counts per minute from duplicate cultures. The standard error of the mean for the replicates was less than 15% of the mean.

RESULTS

Expression and purification from E. coli *of recombinant* Fel d *I chains* 1 *and* 2

Fel d I chain 1 and chain 2 cDNAs had an oligonucleotide sequence encoding a thrombin cleavage site (LVPR↓GS) added to each of their 5′ ends using PCR methods (Fig. 1A and B). A polyhistidine encoding sequence was also added to facilitate purification using Ni^{2+} metal-ion affinity chromatography [Fig. 1C (Hochuli *et al.*, 1988)]. The resulting expression cassette was cloned into the pTrc99 (Amann *et al.*, 1988) and pET11d vectors (Studier *et al.*, 1990) and expression of the rchain 1 and rchain 2 fusion proteins was induced by IPTG (Fig. 1C). The pTrc99 vector contains a strong IPTG-inducible hybrid *trp/lac* promoter adjacent to the cDNA insertion site. Although this vector has been successfully used to recombinantly produce the allergens *Amb a* I.1 and *Amb a* II (Bond *et al.*, 1991; Rogers *et al.*, 1991), this *trp/lac* promoter system did not yield high levels of mRNA encoding chain 1 and chain 2 or appreciable accumulated recombinant products (data not shown). In contrast, expression using the T7 polymerase based pET-11 vector in conjunction with purification on NTA-Agarose (Hochuli *et al.*, 1988) of rchain 1 and rchain 2, led to reproducible high yields with a purity exceeding 90% in a single step as assessed by densitometric analysis of a Coomassie Blue stained SDS–PAGE gel (Fig. 2A and B; lane 5; data not shown). Recombinant chain 1 has an apparent M_r of approximately 10 kDa as predicted for the 70 amino acid chain 1 plus 30 non-relevant residues at the *N*-terminus, 16 of which are derived from T7 gn 10 (Fig. 3A). Recombinant chain 2 has the expected apparent M_r of approximately 12 kDa as predicted for the 92 amino acid chain 2 plus the same 30 non-relevant residues at the *N*-terminus (Fig. 3B). The pET-11d expression system and the Ni^{2+} metal-ion-affinity chromatography method usually generates approximately

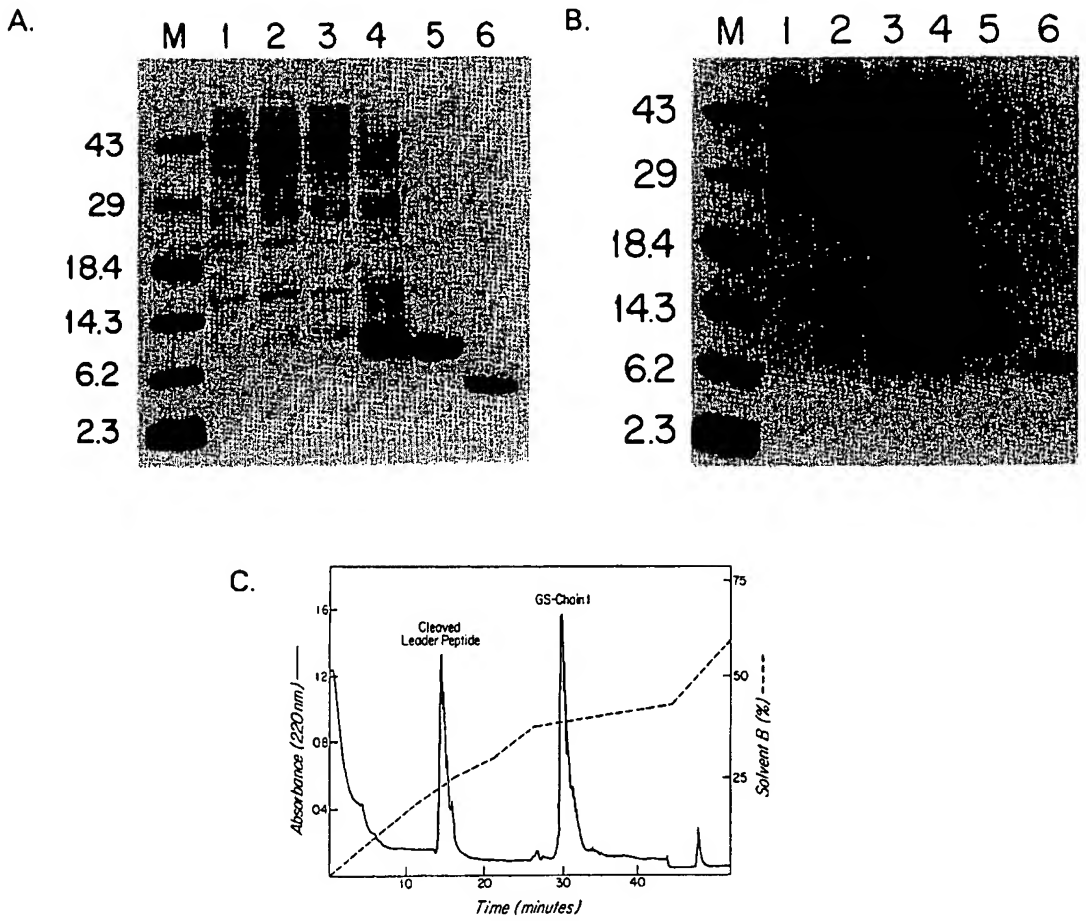

Fig. 2. Expression and purification of rchain 1 and rchain 2. Samples loaded include: lane M, mol. wt marker standards; lane 1, host strain BL21 lysate; lane 2, BL21 containing expression vector pET11d lysate. In panel A rchain 1 samples were loaded in the following order: lane 3, BL21 containing pET11chain 1 lysate at 0 hr induction with IPTG; lane 4, BL21 containing pET11chain 1 lysate after 2 hr induction with IPTG; lane 5, purified rchain 1 protein after Ni^{2+} metal-ion affinity purification (Hochuli *et al.*, 1988); lane 6, rchain 1cp after thrombin cleavage of the *N*-terminal leader peptide and separation by reverse-phase HPLC purification. In panel B, lanes 3–6 were as for panel A except that rchain 2 equivalent samples were loaded. In both A and B, lanes 1–4, equivalent amounts of protein were loaded and therefore the level of expression can be estimated by Coomassie blue staining of an 18% SDS–polyacrylamide gel. In panel C, a trace of thrombin cleaved rchain 1 chromatographically separated on reverse-phase HPLC is represented. The rchain 1cp is well resolved on this system from the 28 aa *N*-terminal cleavage fragment.

40 mg rchain 1 per l of growth medium and 20 mg/l of rchain 2.

Thrombin cleavage and isolation of highly purified Fel d *I rchain* 1 *and rchain* 2

After expression in *E. coli* and metal-ion affinity chromatography, rchain 1 and rchain 2 possess non-relevant sequences at their *N*-terminal ends (Fig. 3A and B). This 30 amino acid stretch is composed of the *N*-terminal amino acids of the T7 gn 10 major coat protein (MASMTGGQQMGRISMG), the polyhistidine purification tag $(H)_6$, the amino acids EF encoded by the *Eco*R I cloning site and an introduced thrombin recognition site (LVPR↓GS) (Chang, 1985). Cleavage with thrombin permitted the removal of all the non-relevant *N*-terminal residues with the exception of the remaining GS part of the thrombin recognition sequence (Fig. 3A and B). The intact rchains 1 and 2 were purified by reverse-phase HPLC after thrombin cleavage. These products, with the only non-relevant *N*-terminal sequence being GS, are designated rchain 1cp and rchain 2cp, respectively. The *N*-terminal 28-residue cleavage product was well resolved from the rchain 1cp and rchain 2cp by HPLC (Fig. 2, panel C). The cleavage and recovery efficiency was >80% for rchain 1cp and approximately 60% for rchain 2cp. The rchain 1cp, a polypeptide chain comprised of 72 amino acids, has an apparent M_r of 5-kDa which is smaller than expected (Fig. 3A, lane 6). Interestingly, this same anomalous M_r was also observed previously on SDS–PAGE analysis of the native allergen (Morgenstern *et al.*, 1991; Duffort *et al.*, 1991).

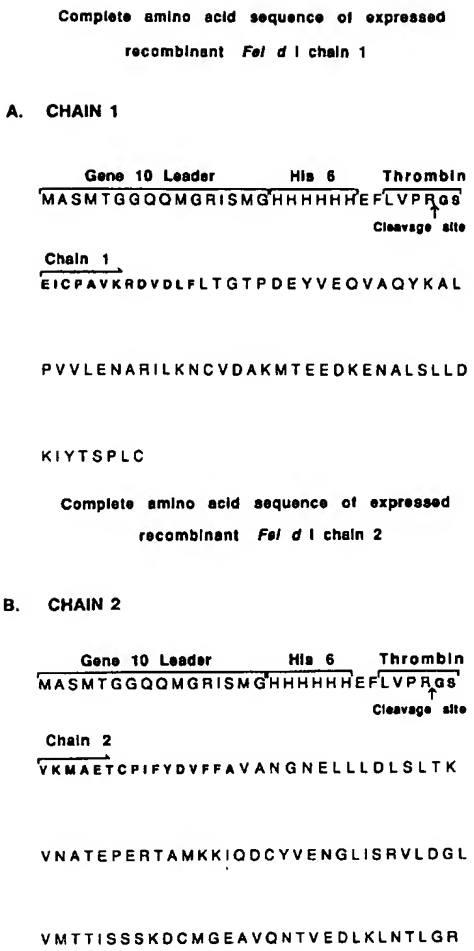

Fig. 3. Complete amino acid sequence of expressed rchain 1 and rchain 2. The complete amino acid sequence of rchain 1 (panel A) and rchain 2 (panel B) is shown in the single letter code. The gene-10 leader sequence, the six sequential histidines used for the Ni^{2+} metal-ion affinity purification (Hochuli *et al.*, 1988) and the introduced thrombin cleavage site are all shown in square brackets. The mature chain 1 and chain 2 protein *N*-termini are shown by horizontal arrows. The thrombin cleavage site is shown by a vertical arrow. The rchain 1cp and rchain 2cp polypeptides were subjected to *N*-terminal sequencing and the residues detected are shown in small bold type. The chain 2 sequence is of the long form (Griffith *et al.*, 1992).

Structural analysis of rchain 1cp and rchain 2cp

The reverse-phase HPLC purified rchain 1cp and rchain 2cp were subjected to *N*-terminal sequencing analysis. This analysis showed that the *N*-termini of these highly purified chains started with the expected residues, GS, and continued into the appropriate translated sequence of *Fel d* I chain 1 and chain 2 (Fig. 3A and B, respectively). This result demonstrated the integrity of the thrombin cleavage reaction and verified that the rchain 1cp and rchain 2cp *N*-termini are intact and not structurally heterogeneous. Samples of rchain 1cp and rchain 2cp were subjected to mass spectrometry. The molecular ions of rchain 1cp and rchain 2cp have mass numbers of 8004 and 10,266, respectively. These observed mol. wts are within 0.2% of the calculated theoretical mass numbers of 8016 and 10,277, respectively.

Binding of cat-allergic patients' IgE to rchain 1cp and rchain 2cp

Direct-binding ELISA methods were used to determine the *Fel d* I-specific IgE binding capacity of the two recombinant *Fel d* I chains. No detectable difference in IgE binding was observed between the uncleaved rchains 1 and 2 and the rchain 1cp and rchain 2cp, suggesting that the presence of 30 non-relevant residues at the *N*-terminus does not significantly effect the binding properties of these polypeptides (data not shown; see Fig. 3A and B). Figure 4A shows the binding of pooled cat-allergic human IgE to affinity purified *Fel d* I (Chapman *et al.*, 1988), rchain 1cp, and rchain 2cp. The same assay was performed with plasma from several individual patients and a representative example is shown in Fig. 4B (patient no. 669). Many individuals have reactivity similar to that observed with the sera pool (data not shown). Although this assay is not a highly quantitative method, it is clear that human cat-allergic IgE binds specifically to both rchain 1cp and rchain 2cp. Most cat-allergic patients have specific IgE that binds to each polypeptide chain, however, some individual patients' IgE preferentially bind to either rchain 1cp or rchain 2cp in this assay system (data not shown). Experiments are presently underway to quantitatively determine in a large panel of individual patients what proportion of IgE specific for *Fel d* I can bind to these recombinant chains. Preliminary experiments suggest a range of 30–50% of native *Fel d* I-specific IgE can recognize rchain 1cp and rchain 2cp (Bond, manuscript in preparation).

Human T cells from cat-allergic patients react to rchain 1cp and rchain 2cp

T cell lines from two cat-allergic patients were established using affinity purified *Fel d* I (Chapman *et al.*, 1988) as the priming Ag. The cell lines were cultured in the presence of IL-2 and IL-4 until "rested" and no longer responsive to growth factors. At this time the cultures were set up in secondary proliferation assays. As shown in Fig. 5, T cells from these two cat-allergic patients proliferate in response to the priming Ag, native affinity purified *Fel d* I (Chapman *et al.*, 1988), as well as rchain 1cp and rchain 2cp. This result demonstrates that each polypeptide chain of the *Fel d* I heterodimeric protein significantly contributes to the overall T cell response. Although these proliferation data are not quantitative, many patients' T cells respond more vigorously to rchain 1cp than to rchain 2cp, suggesting that chain 1 may harbor a larger proportion of T cell epitopes than chain 2. Using these and other similar T cell lines established from cat-allergic patients, overlapping sets of chain 1 and chain 2 peptides have been screened for a clearer definition of the major regions of T cell recognition of this molecule (Garman *et al.*, submitted).

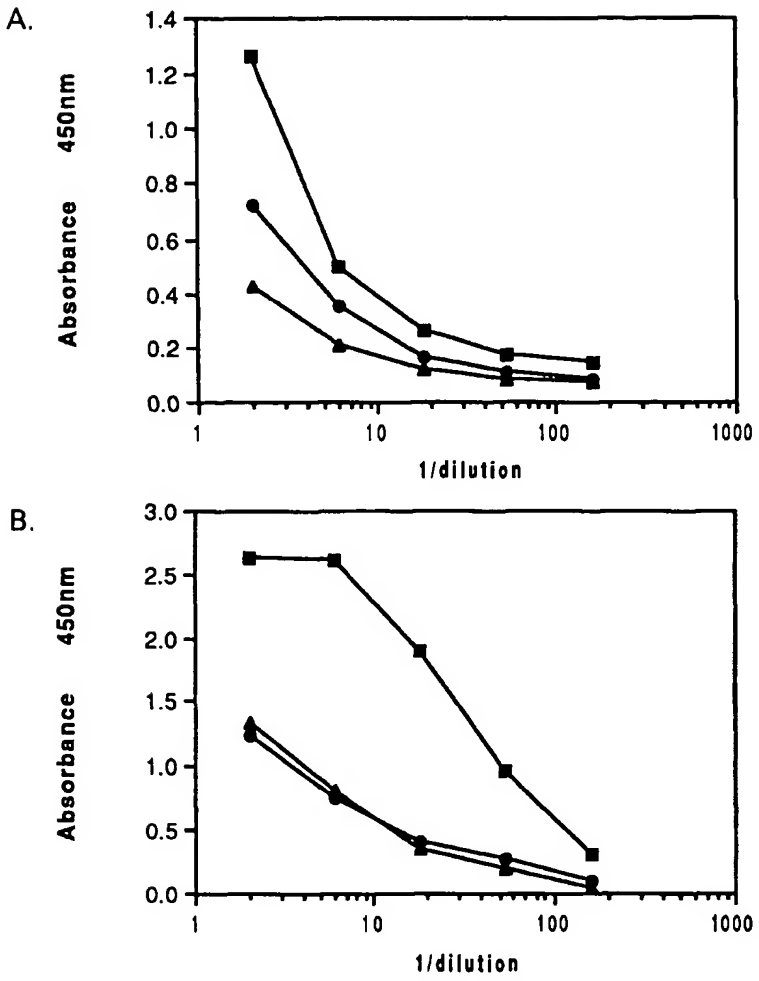

Fig. 4. Direct binding ELISA assays of the reaction between immobilized preparations of rchain 1cp and rchain 2cp and cat-allergic human IgE. Different dilutions of a pooled plasma from 20 cat-allergic patients (panel A), or a single cat-allergic patient No. 669 plasma (panel B), were added in duplicate to antigen-coated wells. The antigens added include: affinity purified *Fel d* I (■); rchain 1cp (▲); and rchain 2cp (●). The *Fel d* I specific IgE binding was measured by the addition of biotinylated goat anti-human IgE plus horseradish peroxidase-conjugated streptavidin followed by a TMB-based chromatographic assay.

DISCUSSION

In the last several years the primary structure of a number of important allergens have been defined by cDNA cloning, including *Der p* I (Chua *et al.*, 1988), *Der p* II (Chua *et al.*, 1990), *Bet v* I (Breiteneder *et al.*, 1989), *Amb a* I (Rafnar *et al.*, 1991), *Amb a* II (Rogers *et al.*, 1991), *Lol p* I (Perez *et al.*, 1990; Griffith *et al.*, 1991), *Poa p* IX (Olsen *et al.*, 1991), *Lol p* IX (Singh *et al.*, 1991) and *Fel d* I (Morgenstern *et al.*, 1991; Griffith *et al.*, 1992). These various cDNA have been expressed using several different expression systems, most of which utilize a fusion-protein based approach (Chua *et al.*, 1991; Mohapatra *et al.*, 1990). One disadvantage of several of these systems is that the fusion portion of the recombinant molecule may constitute a substantial proportion of the product, presenting difficulties in the isolation in high yield of the recombinant allergen polypeptide free of irrelevant sequence. The T7 promoter/T7 RNA polymerase based pET-11d expression system has been successful for the production of proteins that are toxic to *E. coli* or expressed at low levels with other expression systems (Bianchi, 1991). The system described herein, the use of the high-level expression vector pET-11d (Studier *et al.*, 1990) in conjunction with Ni^{2+} metal-ion affinity chromatography (Hochuli *et al.*, 1988), has allowed the expression of rchain 1 and rchain 2 of *Fel d* I with both a high yield and a high level of purity (Fig. 2).

Recombinant chain 1 and rchain 2 have been produced as fusion proteins with 30 aa of non-relevant sequence at the *N*-terminus (Fig. 3). This non-relevant *N*-terminal sequence constitutes 30% by weight of the rchain 1 (30 aa out of 100 aa) and ~25% by weight of rchain 2 (30 aa out of 122 aa). The addition of the gn 10 leader sequence to these recombinant proteins increased the expression level of the rchain 1 approximately two–three fold, whereas no change in expression level

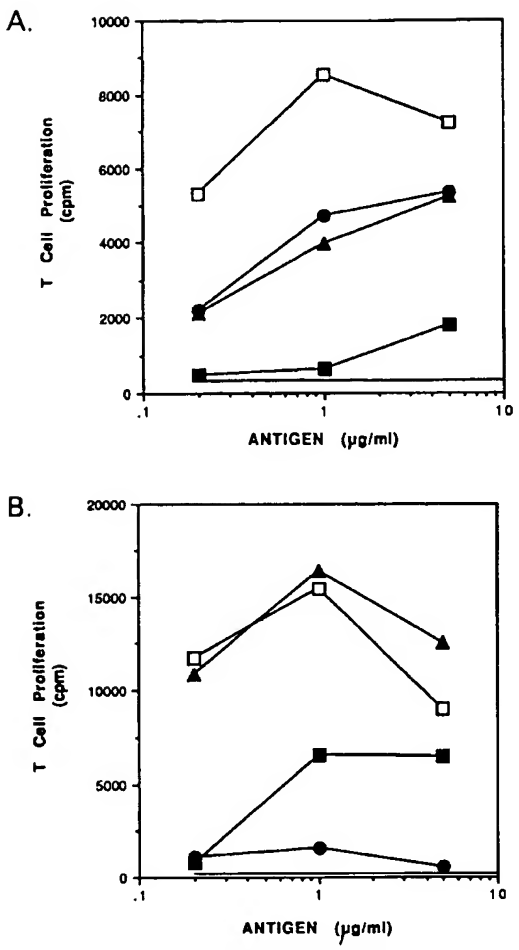

Fig. 5. Human T cell responses to affinity purified *Fel d* I, rchain 1cp and rchain 2cp. PBL from cat-allergic patients No. 1074 (panel A) and No. 1092 (panel B) were cultured in the presence of affinity purified *Fel d* I (Chapman *et al.*, 1988), rested, and restimulated in secondary assays with the antigens indicated: medium only (—); affinity purified *Fel d* I (■); rchain 1cp (▲); rchain 2cp (●); a mixture of rchain 1cp and rchain 2cp (□). The proliferation of the T cells was assessed by the uptake of tritiated thymidine in triplicate cultures.

was observed with rchain 2 (data not shown). The introduction of a thrombin cleavage site by PCR methods (Fig. 1) allowed the cleavage and HPLC purification of each of these chains almost free of extraneous sequence (only GS, part of the thrombin recognition sequence, remains at the *N*-terminus). Laser-desorption mass spectrometry of rchain 1cp and rchain 2cp verified that these two purified polypeptide chains are of the appropriate mol. wt. Also, *N*-terminal sequence analysis demonstrated that rchain 1cp and rchain 2cp have the expected *N*-terminus and are not structurally heterogeneous. Not all cleavage methods are equally effective since a Factor Xa recognition site was also introduced into the same expressed product but the presence of significant cryptic internal cleavage sites within both rchain 1 and rchain 2 complicated purification and also resulted in a lower yield (data not shown). In summary, these methods allowed the generation of highly purified, structurally intact, rchain 1cp and rchain 2cp that can be effectively used in the molecular analysis of IgE reactivity or as Ag in T cell studies.

The *Fel d* I molecule is the only allergen known so far to be a heterodimeric protein derived from two independent gene products (Morgenstern *et al.*, 1991; Griffith *et al.*, 1992). Therefore, it is interesting to determine whether one or both of the two chains that constitute *Fel d* I is primarily responsible for the allergenicity of the whole molecule. Physicochemical characterization of the native affinity purified *Fel d* I (Chapman *et al.*, 1988), has established that chain 2 is a glycopeptide with *N*-linked oligosaccharides (Morgenstern *et al.*, 1991; Duffort *et al.*, 1991). IgE binding studies with *Fel d* I that had been treated with the deglycosylating enzyme, peptide *N*-glycosidase-F (Morgenstern *et al.*, 1991), demonstrated that this deglycosylation treatment did not significantly reduce IgE binding. However, it is difficult to assess whether this enzymatic treatment has completely deglycosylated the protein. It is also very difficult to quantitatively recover the individual chains of this molecule for study. Expression of the rchain 1 and rchain 2 in *E. coli*, a glycosylation deficient host, permits a more definitive examination of the importance of glycosylation with respect to anti-*Fel d* I specific IgE binding. The suggestion that considerable IgE binding is directed to the polypeptide backbone and not significantly dependent on the presence of carbohydrate was confirmed by the detection by direct binding ELISA of substantial IgE reactivity to rchain 1cp and rchain 2cp (Fig. 4). Therefore, rchain 1cp and rchain 2cp could be very valuable reagents in standardizing ELISA measurements of *Fel d* I exposure in house dust samples and commercial cat allergen extracts.

The exact conformation of the native *Fel d* I is not known. When native *Fel d* I is subjected to SDS–PAGE analysis under reducing conditions, chain 1 has an apparent M_r of 5-kDa which is lower than that predicted by its primary sequence of 70 aa (Duffort *et al.*, 1991; Morgenstern *et al.*, 1991). The rchain 1cp also exhibits a lower apparent M_r than expected (Fig. 2A, lane 6). Since the *N*-terminal sequencing and mass spectrometry data demonstrated that the rchain 1cp is structurally intact (Fig. 3), this apparent M_r (5-kDa) is most likely due to rchain 1cp forming a stable folded protein configuration even in the presence of a high concentration of SDS. Although the biological function of *Fel d* I is not known, chain 1 has detectable sequence homology with rabbit uteroglobin (Morgenstern *et al.*, 1991) and this relatedness extends to their genomic organization (Griffith *et al.*, 1992). Recombinant uteroglobin readily folds into its appropriate secondary structure when expressed in *E. coli*, and maintains biological activity (Miele *et al.*, 1990). Taken together, these data suggest that rchain 1cp may also readily refold into its native conformation and may explain its anomolous gel migration. Experiments are presently underway to refold the isolated rchain 1cp and rchain 2cp into a heterodimeric structure resembling the native *Fel d* I.

The generation of highly purified rchain 1cp and rchain 2cp permitted the examination of the cat-allergic human T cell reactivity to each chain of *Fel d* I. T cell lines established using affinity purified *Fel d* I (Chapman *et al.*, 1988) as the priming Ag, were examined in *in vitro* secondary stimulation assays for reactivity to rchain 1cp or rchain 2cp (Fig. 5). It is clear from these data that the cat-allergic human T cell response is directed against both chain 1 and chain 2 of *Fel d* I. Overlapping peptides, based on the primary structure of *Fel d* I (Morgenstern *et al.*, 1991; Griffith *et al.*, 1992), have recently been used to map the T cell epitopes in *Fel d* I. The proliferation data using rchain 1cp and rchain 2cp (Fig. 5) and the epitope mapping using synthetic peptides (Garman *et al.*, submitted) are consistent with chain 1 contributing more than chain 2 to the overall T cell reactivity. It has recently been proposed that the administration of peptides encoding T cell epitopes may offer a new therapeutic approach to allergy desensitization (O'Hehir *et al.*, 1991; Schad *et al.*, 1991; Gefter, 1992). Highly purified rchain 1cp and rchain 2cp, may be valuable reagents in this endeavor. This last possibility is supported by recent experiments in mice that show a reduced *in vitro* response to *Fel d* I after prior exposure *in vivo* to rchain 1cp (Briner *et al.*, submitted).

In summary, the availability of highly purified rchain 1cp and rchain 2cp of *Fel d* I permits a detailed examination of the IgE reactivity to this important allergen. These reagents will also play an important role in studies to understand the human T cell response to the *Fel d* I allergen.

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Immunology

Amino acid sequence of Fel dI, the major allergen of the domestic cat: Protein sequence analysis and cDNA cloning

(polymerase chain reaction/N-glycosylation)

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ABSTRACT **The complete primary structure of Fel dI (International Union of Immunological Societies nomenclature), the major allergen produced by the domestic cat, *Felis domesticus*, was determined by protein sequence analysis and cDNA cloning. Protein sequencing of Fel dI from an immunoaffinity-purified extract of house dust revealed that the allergen is composed of two polypeptide chains. Degenerate oligonucleotides derived from the protein sequence were used in polymerase chain reaction amplification of cat salivary gland cDNA to demonstrate that the two chains are encoded by different genes. Chain 1 of Fel dI shares amino acid homology with rabbit uteroglobin, while chain 2 is a glycoprotein with N-linked oligosaccharides.**

The house cat (*Felis domesticus*) is a significant source of proteins known to elicit the symptoms of allergic disease. These symptoms range in severity from the relatively minor discomforts of rhinitis and conjunctivitis to potentially life-threatening asthmatic episodes. The frequency of cat allergy in the United States is on the order of 10% (1). A substantially greater proportion (20–30%) of asthmatics respond with immediate hypersensitivity upon skin test challenge with cat allergens (2, 3), and this hypersensitivity is a significant risk factor associated with the disease (4, 5).

Although allergic patient serum reacts with a number of proteins found in cat pelt extracts (6, 7), the dominant allergen is Fel dI (ref. 8; International Union of Immunological Societies nomenclature; ref. 9). Specifically, radioallergosorbent test (RAST) and crossed immunoelectrophoresis (CIE)/crossed radioimmunoelectrophoresis (CRIE) analysis of IgE antibodies in the serum of allergic patients have shown that the majority of this antibody response is directed against Fel dI (7, 10–13). The clinical relevance of these findings has been reinforced by studies detecting Fel dI-specific IgE in the serum of at least 80% of cat-allergic patients (6, 7, 10). Airborne levels of Fel dI found in houses with cats are often in excess of those required to provoke an asthmatic response in experimental aerosol bronchial challenge (14, 15). In addition, cat-allergic patients administered partially purified Fel dI in immunotherapy regimens experienced significantly diminished bronchial reactivity upon antigen challenge relative to control patients (16, 17).

Fel dI, found in the saliva, sebaceous glands, and pelts of cats (18–20), has been purified via biochemical (12) and immunoaffinity techniques (13, 21) and characterized as an acidic glycoprotein with a molecular mass of approximately 38 kDa. Under reducing conditions the apparent molecular mass of Fel dI shifts to ≈18 kDa, implying a multimeric structure for the antigen (12, 22).

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T-cell recognition is believed to play a pivotal role in the immunological response to allergens. Upon activation by an allergen, T cells secrete cytokines whose effects include both the activation of inflammatory cells and the supply of help to B cells for IgE production (23). T-cell epitopes within a protein are, for the most part, defined by the primary sequence of short peptides derived from intact protein. It is this primary sequence that determines the nature of the interaction of a peptide with the T-cell receptor and HLA molecules (24–26). Hence, the goal of the work presented in this manuscript was to define the primary structure of Fel dI.§ Such knowledge should aid the study and treatment of the human allergic response to cats.

MATERIALS AND METHODS

Immunoblot Analysis. Gel electrophoresis and electroblot transfer/immunodetection were performed as described (27).

Biotinylated goat anti-rabbit immunoglobulin (Southern Biotechnology Associates, Birmingham, AL) or biotinylated goat anti-human IgE (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used as the second antibody, and ^{125}I-labeled streptavidin (Amersham) was used as a reporter ligand for autoradiography at −80°C with intensifying screen.

Histamine Release Analysis. Release of histamine from basophils was measured with a RIA kit (AMAC, Westbrook, ME) with a monoclonal antibody (mAb) specific for an acylated derivative of histamine (28).

Protein Sequence Analysis. A 10% (wt/vol) aqueous extract of dust collected from a house with four pet cats was affinity-purified via a mAb anti-Fel dI column (21). Sequence analyses were performed with an Applied Biosystems 477A protein sequenator connected to an on-line phenylthiohydantoin analyzer. N-terminal sequence analysis of intact Fel dI was performed after concomitant pretreatment of the protein sample *in situ* with tributylphosphine and 4-vinylpyridine, giving repetitive yields > 93% (29). *o*-Phthalaldehyde (OPA) (30) was applied to block N termini except those with prolines (positions 4 and 32 of chain 1 or positions 7 and 37 of chain 2).

Internal sequence was determined by proteolytic digestion of intact protein. Reduced and pyridylethylated Fel dI was digested with endopeptidase Lys-C, endopeptidase Asp-N, or endoproteinase Glu-C (Boehringer Mannheim) or was cleaved with 2% (wt/vol) CNBr in 70% (vol/vol) formic acid (31) overnight at room temperature. Time dependent *in situ* CNBr digestion of Fel dI on the sequencer glass filter disk (32) was performed after five sequencer cycles, preventing

Abbreviations: Fel dI, *Felis domesticus* allergen 1; OPA, *o*-phthalaldehyde; PCR, polymerase chain reaction; mAb, monoclonal antibody.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession nos. M74952, M74953, and M77341).

the formation of a CNBr fragment between Met-3 and Met-42 of chain 2, after which the sample was blocked with acetic anhydride to reduce spurious sequence signals. Alternatively, tryptic peptides were obtained by enzymatic digestion (Worthington) of unmodified Fel dI. The peptides were separated on an Applied Biosystems 151A HPLC system with an Aquapore RP300 column and subjected to sequence analysis.

Anti-Peptide Antisera. Peptides Fel 1 (chain 1, residues 1–17), Fel 2 (chain 1, residues 9–25), Fel 4 (chain 2, residues 37–55), and Fel 18 (chain 2, residues 23–48) were made with Applied Biosystems 430 or Milligen 9050 peptide synthesizers. Peptides were conjugated to keyhole limpet hemocyaninin via ethyldicyclohexylcarbodiimide and injected into rabbits with complete Freund's adjuvant, followed by one booster. Anti-peptide antibodies were affinity-purified by absorption/elution with cognate peptide-Sepharose columns.

RNA Preparation and cDNA Synthesis. Mandibular and parotid glands from five cats (BioMedical Associates, Friedensburg, PA) were pooled and frozen in liquid N_2. Total RNA was prepared by grinding the frozen tissue in guanidine thiocyanate and forming a pellet through a CsCl cushion (33). cDNA was synthesized using Superscript reverse transcriptase (Bethesda Research Laboratories). Second-strand cDNA synthesis (34) was carried out with a kit reaction mixture from Bethesda Research Laboratories.

Polymerase Chain Reaction (PCR). An MJ Research Programmable Thermal Controller was used in PCR amplification of cDNA with primers (Oligos Etc., Guilford, CT and Research Genetics, Huntsville, AL) and a GeneAmp kit (Perkin–Elmer/Cetus). Reactions proceeded for 30 cycles of 1 min at 94°C, 1.5 min at 55°C, and 1 min at 72°C for exact primers or five cycles of 1 min at 94°C, 1.5 min at 45°C, and 1 min at 72°C, followed by 25 cycles of 1 min at 94°C, 1.5 min at 55°C, and 1 min at 72°C for degenerate primers. A 1/100th aliquot of an initial reaction was used in sequential rounds of PCR with nested primers.

DNA Subcloning and Sequencing. Standard protocols were used for recombinant DNA manipulations (35). Amplified PCR products were digested with restriction enzymes, isolated from 2–3% agarose gels (NuSieve; FMC), and ligated into Bluescript (Stratagene) or phage M13mp18/19 replicative form vectors. DNA sequence analysis (36) was performed with a Sequenase 2.0 kit (United States Biochemical). Asymmetrically amplified PCR products (37) were purified with Qiagen 5 tips (Diagen GmbH) prior to sequencing.

Sequence Homology Analysis. A sequence homology database search was performed with the PROSEARCH program (38) on an AMT 600 distributed array processor (Active Memory Technology, Reading, U.K.).

Glycosidase Treatment. Two milligrams of Fel dI were boiled for 2 min in 0.2% SDS, 5% (vol/vol) 2-mercaptoethanol, cooled to room temperature, and incubated with 6 units of peptide N-glycosidase F in 50 mM KH_2PO_4, pH 7.1/1% (wt/vol) octyl glucoside (Boehringer Mannheim) for 17 hr at 37°C.

RESULTS

An aliquot of immunoaffinity-purified house dust extract (21) was tested for human allergenic activities *in vitro* prior to protein sequence analysis. Immunoblot analysis of the immunoaffinity-purified protein was performed to assess its IgE-binding capacity. Native protein was subjected to isoelectrophoretic focusing in a pH gradient between 2.5 and 5.0, electroblotted onto nitrocellulose, and probed with the anti-Fel dI mAb 1G9 (21) or with serum from one cat-allergic patient and one nonallergic patient (as determined by skin test challenge with cat pelt extracts). The immunoreactivity of the cat-allergic patient's serum to the native purified protein was readily apparent in contrast to that of the nonallergic patient's serum (Fig. 1*A*). In addition, the isoelectrophoretic mobilities of the molecules recognized by the cat-allergic patient's serum and the anti-Fel dI mAb were identical and approximate the previously reported pI for Fel dI of 3.85 (12). Similarly, only the IgE from cat-allergic patient's serum possessed reactivity to the reduced and denatured purified proteins on the SDS/PAGE immunoblot (Fig. 1*B*).

Blood cells collected from the allergic and nonallergic patients were incubated with various dilutions of the immunoaffinity-purified house dust extract, and the levels of histamine released from basophils were quantitated. Concentrations of purified protein as low as 100 ng/ml elicited a strong response from the cat-allergic patient's basophils, whereas 1000-fold higher levels of protein failed to mediate release of histamine from the nonallergic patient's basophils (Fig. 1*C*). As a positive control, antibody-mediated crosslinking of surface-bound IgE on both patients' basophils provoked equivalent levels of histamine release.

The data from the above *in vitro* analyses strongly suggest that the major species in the purified extract, presumably Fel dI, is allergenic in nature. Moreover, these results are representative data from experiments involving 10 patients (data not shown).

A 2-mg aliquot of the immunoaffinity-purified protein was used for sequence determination. N-terminal sequence analysis of intact protein revealed a major amino acid sequence in conjunction with several minor ones. The major sequence (Fig. 2*A*), which corresponded to the published N-terminal 33 residues of Fel dI (21) with the exception of two residues, was termed chain 1. The most prevalent minor sequence (present at 55% of the major sequence level) was designated chain 2 (Fig. 2*B*). All of the remaining minor sequences could be accounted for as having arisen from N-terminal proteolytic deletions of chain 2. Summation of the sequence signals

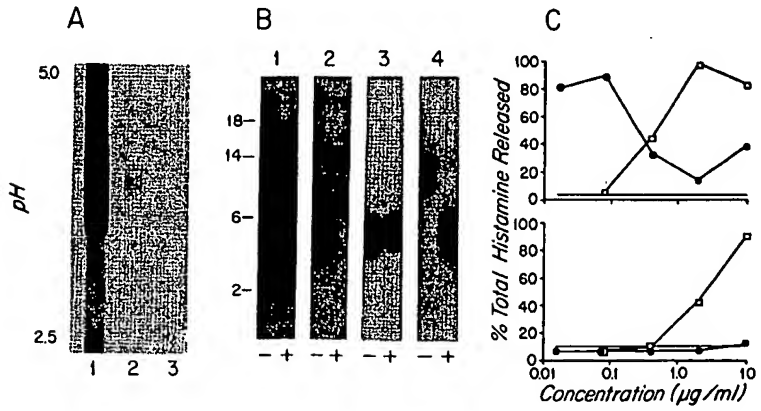

FIG. 1. *In vitro* allergenic activity of immunoaffinity-purified Fel dI. (*A*) Isoelectric focusing/immunoblot analysis of Fel dI with the following primary antibodies in lanes: 1, 1G9 anti-Fel dI mAb; 2, cat-allergic patient serum; 3, nonallergic patient serum. (*B*) SDS/PAGE/Western blot analysis of untreated Fel dI (lanes −) and peptide N-glycosidase F-treated Fel dI (lanes +) with the following primary antibodies in lanes: 1, cat-allergic patient serum; 2, nonallergic patient serum; 3, chain 1 Fel dI anti-peptide antisera; 4, chain 2 Fel dI anti-peptide antisera. Sizes are shown in kDa. (*C*) Fel dI-mediated histamine released from cat-allergic (*Upper*) and nonallergic (*Lower*) patient's basophils. Solid line with no symbols, buffer control; ●, Fel dI; □, HP6061 mouse anti-human IgE mAb.

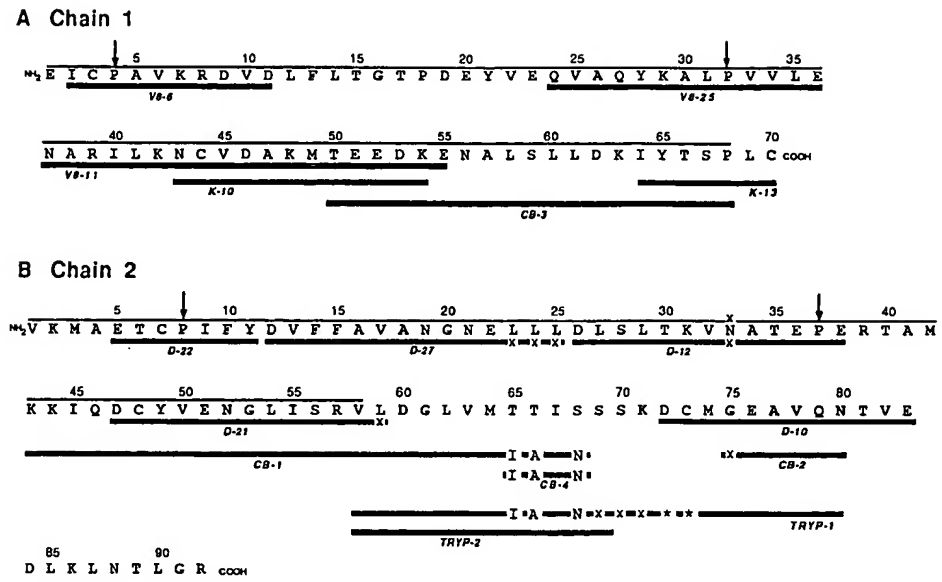

FIG. 2. Protein sequence analysis of Fel dI. (*A*) Chain 1 of Fel dI. (*B*) Chain 2 of Fel dI. The overlined sequence was determined from intact Edman degradation. The underlined sequences were determined from proteolytic fragments generated from digestion by: V8, *Staphylococcus aureus* V8; K, endopeptidase Lys-C; D, endoproteinase Asp-N; TRYP, trypsin; and CB, CnBr. x, Undetermined residue; ⋆, space in sequence. Arrows mark the site of blockage by OPA treatment.

indicated that chains 1 and 2 were present at equimolar ratios in the Fel dI preparation.

Since residues 4 of chain 1 and 7 of chain 2 were determined to be proline, OPA (which modifies primary but not secondary amines, such as proline) blockage was used to perform isolated sequence analysis of each of the two chains of Fel dI. Applying OPA before the fourth cycle of Edman degradation blocked the N terminus of chain 2, allowing unambiguous sequence data to be obtained for chain 1. Similarly, to acquire chain 2 sequence, OPA was used prior to the seventh sequencing cycle to block chain 1 (note the arrows at proline residues in Fig. 2). OPA blockage at these cycles, as well as at cycle 32 for chain 1 and cycle 37 for chain 2, permitted significant extension of the N-terminal protein sequence for chain 1 (68 amino acid residues) and chain 2 (58 amino acid residues).

Intact N-terminal protein sequence was confirmed and extended by sequence analysis of peptides derived from enzymatic and chemical digests of the purified Fel dI (Fig. 2). After *in situ* CNBr digestion, one major peptide sequence, CB-1 and three minor peptides that were present at 60% (CB-2), 38% (CB-3), and 12% (CB-4) of the signal level of the major peptide were identified. CB-1 was 25 amino acids in length, with an N terminus corresponding to residue 43 of chain 2, thereby extending the sequence of chain 2 to 68 residues.

Sequence from the tryptic peptide TRYP-1 and the endoprotease Asp-N-derived peptide D-10 overlapped and, when applied in conjunction with the CNBr peptides CB-1 and CB-2, extended the sequence of chain 2 to 83 residues (Fig. 2*B*). Similarly, K-13, an endopeptidase Lys-C-generated peptide, was contiguous with residues 64–68 of chain 1 and possessed two additional residues; this indicated chain 1 is comprised of at least 70 amino acids (Fig. 2*A*).

Two incongruities became apparent during protein sequence analysis of Fel dI. When compared to peptide TRYP-2, peptides CB-1, TRYP-1, and D-10 revealed sequence and size polymorphism between residues 65 and 73 of chain 2 (Fig. 2*B*). In addition, sequence signal at residue 33 of chain 2 was not detected during intact or peptide-derived sequence analysis.

Partial cDNAs encoding portions of Fel dI chains 1 and 2 were obtained in three discrete PCR amplifications. Parotid and mandibular glands were used as a source of Fel dI mRNA, since cat saliva harbors high levels of the allergen (7). By using degenerate 5′ sense and 3′ antisense primer pairs (Fig. 3) based on contiguous amino acids of limited codon ambiguity (39), internal portions of chains 1 and 2 were amplified from parotid/mandibular cDNA. DNA fragments of the predicted size were subcloned and sequenced (Fig. 4). The deduced amino acid sequence of the clones confirmed the PCR products' authenticity as portions of the cDNAs for chains 1 and 2 of Fel dI.

The 5′ and 3′ portions of the chain 1 and chain 2 cDNAs were obtained by anchored PCR methods. Two consecutive rounds of PCR amplification were carried out with a nested pair of chain 1- or chain 2-specific 5′ primers in conjunction with a 3′ primer (ED primer, Fig. 3) encoding a tag sequence covalently linked 3′ to the oligo(dT) primer (EDT primer, Fig. 3) used in first-strand cDNA synthesis (40). These cDNA fragments encompassed the 3′ ends of chains 1 and 2, including stop codons, 3′ untranslated sequences, polyadenylylation signals, and poly(A) tracts (Fig. 4).

Similarly, the 5′ regions of the cDNAs were isolated after ligating an "anchor" template (41) onto the 5′ termini of double-stranded parotid/mandibular cDNA; this anchor sequence (42) was then used as a 5′ primer in successive PCR amplifications with a nested pair of chain 1- or chain 2-spe-

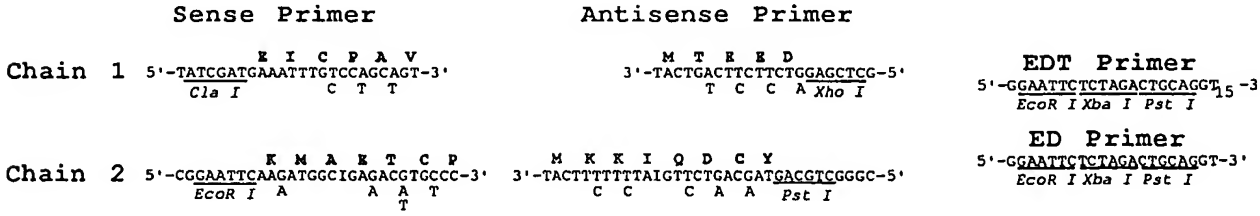

FIG. 3. Primers and probes used to amplify by PCR and subclone internal and 3′ portions of cDNAs of Fel dI chains 1 and 2.

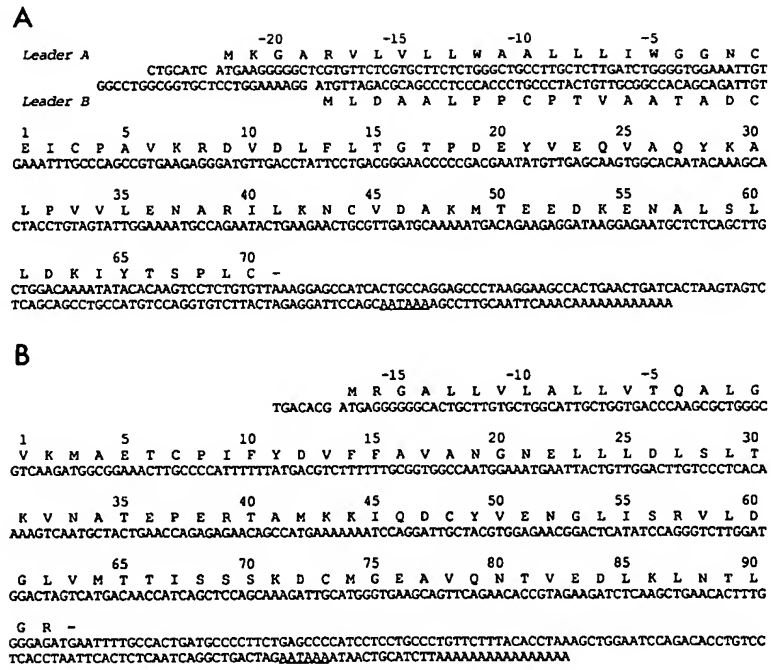

FIG. 4. cDNA sequence of Fel dI. Negative residues compose the leader sequences, and residue 1 represents the amino terminus of the mature proteins. Polyadenylylation signals are underlined. (*A*) Chain 1 of Fel dI including both leader sequences. (*B*) Chain 2 of Fel dI.

cific 3′ primers. Two different 5′ partial cDNAs were identified for chain 1 (leaders A and B in Fig. 4*A*) in contrast to a single one for chain 2 (Fig. 4*B*). Each of the three 5′ partial cDNAs had open reading frames with presumptive methionine initiator codons (43).

Contiguous chain 1 and chain 2 Fel dI cDNAs were generated by amplifying parotid/mandibular cDNA with 5′ and 3′ noncoding primer pairs. These PCR products were reamplified asymmetrically (38) to generate single-stranded template for sequence analysis; direct sequencing of the PCR products confirmed the sequence for chains 1 and 2 obtained from the partial cDNAs.

A search of a protein sequence data base for sequence homology with chains 1 and 2 of Fel dI revealed homology between the rabbit uteroglobin precursor (44) and the "full-length" leader A–chain 1 protein (Fig. 5). The observed homology (25 identities and 25 conservative substitutions with two gaps in 93 residues) is such that the probability of two random polypeptides of this size sharing this degree of similarity is less than 1 in 10^4 (38). Homology of a similar extent was found between chain 1 and a 10-kDa protein secreted from human lung Clara cells (45). No proteins with significant homology to chain 2 were identified.

Protein sequence analysis of Fel dI could not identify the amino acid at position 33 of chain 2. The presence of a consensus sequence for N-linked glycosylation (N-A-T at positions 33–35) in Fel dI chain 2 cDNA suggested glycosylation was responsible for the lack of sequence signal at residue 33. To determine whether chain 2 is modified by N-linked glycosylation, Fel dI was treated with peptide N–glycosidase F and analyzed on SDS/PAGE and Western immunoblots with chain 1- or chain 2-specific anti-peptide antisera (Fig. 1*B*). The electrophoretic mobility of chain 2 increased upon digestion with N-glycosidase, while that of chain 1 remained unaffected. This observation and the lack of a consensus sequence for N-linked glycosylation in chain 1 suggest that solely chain 2 of Fel dI is modified by N-linked oligosaccharides.

DISCUSSION

By use of novel techniques, such as OPA blockage and *in situ* acetylation/CNBr cleavage, the greater part of the protein sequence of Fel dI, the major allergen of the domestic cat, was determined. cDNA cloning was then utilized to complete the primary structure analysis of the allergen.

The deduced amino acid sequence of the chain 1 cDNA is in complete agreement with the protein sequence obtained from immunoaffinity-purified Fel dI. cDNA cloning of chain 2 of Fel dI served to link various peptide-derived sequences and reveal an additional nine C-terminal amino acids. However, the sequence and spacing polymorphism detected by protein sequencing in the vicinity of residues 65–73 was not observed in the chain 2 cDNA.

Analysis of the chain 2 cDNA sequence disclosed that residue 33, which could not be resolved by protein sequence

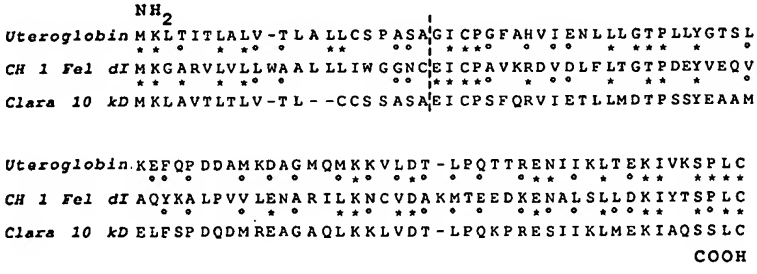

FIG. 5. Protein sequence homology of chain 1 of Fel dI, rabbit uteroglobin, and human Clara cell 10-kDa secretory protein. ☆, Amino acid identities; ∘, conservative amino acid substitutions; vertical dashed line, amino termini of the mature secreted proteins.

analysis, is asparagine. This led to the prediction that chain 2 of Fel dI is a glycoprotein with N-linked oligosaccharides, which was corroborated by an observed increase in the electrophoretic mobility of chain 2 of Fel dI after digestion with Peptide N–glycosidase F. This result is in agreement with a previous report that one of the chains of Fel dI is modified by N-linked oligosaccharides (22).

The isolation of two distinct cDNAs for chains 1 and 2 demonstrates that Fel dI is comprised of polypeptides encoded by different genes. Both cDNAs encode N termini with short stretches of hydrophobic residues, which most likely represent the precursor forms of the individual chains of Fel dI, whereas the N termini determined by protein sequence analysis are probably representative of the mature forms of chains 1 and 2. The two different leaders in the chain 1 cDNAs can be accounted for by alternative splicing of the nascent chain 1 mRNA transcript (unpublished data).

The sequence homology of Fel dI chain 1 with rabbit uteroglobin and the human Clara cell 10-kDa secretory protein is intriguing but inconclusive in establishing a homology of function. All three proteins are synthesized with hydrophobic leader peptides and secreted as mature proteins ≈70 amino acids long. Uteroglobin has been crystallized and shown to exist as a homodimer of antiparallel chains linked by disulfide bonds (46). The 10-kDa secreted protein from human Clara cells is also a disulfide-linked homodimer (45). The tertiary structure of Fel dI appears to be more complex (22), as preliminary analysis indicates chains 1 and 2 are linked by intermolecular disulfide bonds in an antiparallel topology (data not shown).

Uteroglobin is believed to protect the wet epithelia by acting as an immunomodulatory protein, for example, limiting the maternal immune response against the embryo during implantation (47). Uteroglobin possesses both steroid binding (48) and phopholipase A_2 inhibitory (49) activities. The latter activity is shared with lipocortins (50) and mediates an antiinflammatory effect by preventing the formation of arachidonic acid metabolites from membrane phospholipids. Since Fel dI has no known biological function it is interesting to speculate why cat skin epithelia is ubiquitously coated with the protein [either from synthesis *in situ* (20) (unpublished data) or from deposition of salivary proteins during licking]. Perhaps Fel dI is involved in protecting the feline dry epithelia in a manner that is analogous to that by which uteroglobin protects the wet epithelia. Last, if further study demonstrates Fel dI has an inherent ability to modulate immune processes, what role might this activity play in the human allergic response?

The complete primary structure of Fel dI presented in this manuscript should facilitate the mapping of its B- and T-cell epitopes and thereby help to elucidate the mechanism of the human immune response to this clinically important allergen. Ultimately, the knowledge gained from such studies may lead to more effective disease management for cat-allergic patients.

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